0145-6008/94/1801-0164$3.00/0
ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

Vol. 18, No. 1
January/Febuary 1994

Detection of Reduced Acetaldehyde Protein Adducts Using a Unique Monoclonal Antibody

Lynell W. Klassen, Dean J. Tuma, Michael F. Sorrell, Thomas L. McDonald, Jane M. DeVasure, and Geoffrey M. Thiele

Acetaldehyde (AA), the major product of alcohol metabolism, has been shown to bind to proteins in vivo and form chemical adducts. These AA-protein adducts have been shown to alter protein structure and function and may result in tissue damage. Recent reports have shown that polyclonal antibodies can be produced that recognize proteins modified in vitro with AA in the presence of sodium cyanoborohydride ($NaCNBH_3$), a strong reducing (R) agent. Antibodies prepared in this way have been shown to recognize proteins in the livers of rats fed alcohol chronically. Because multiple AA-protein adducts can be recognized by polyclonal antisera, and a variety of adducts may be formed in vitro or in vivo, this study was designed to develop monoclonal antibodies specific for proteins modified by AA. In addition, adducts formed under R conditions are probably chemically different than those formed under nonreducing (NR) conditions, and monoclonal antibodies may provide the specificity required to distinguish these chemical differences. Balb/c mice were immunized with bovine brain tubulin that was modified by treatment with 5 mM AA for 7 days under NR conditions. Sera from immunized animals were tested for antibody activity to the immunogen (protein-NR) and for cross-reactivity to protein-R and unmodified protein. Although the highest serum antibody titers were seen toward the NR adduct, antibodies to the R adduct were also detected. This activity difference was independent of the carrier protein, because NR and R bovine serum albumin, keyhole limpet hemocyanin, and actin also gave similar results when used as the adducted protein. Surprisingly, all the monoclonal antibodies (RT1.1, RT1.2, RT1.3, and RT1.4) produced by hybridomas generated from spleen cells from NR-tubulin immunized mice recognized the R and not the NR adduct. One of these hybridomas (RT1.1) produces an IgG2b antibody that reacts with all tested proteins that have been modified with AA under chemical R conditions. Because of its monoclonal specificity, this antibody may be useful in probing for the presence of R AA-protein adducts made both in vitro and in vivo.

Key Words: Monoclonal Antibody, Acetaldehyde, Alcohol, Adduct, ELISA.

THE CHRONIC CONSUMPTION of alcoholic beverages is a major cause of serious liver disease. Although the etiology of alcohol-related liver disease is largely unknown, much evidence indicates that acetaldehyde (AA), the major breakdown product of ethanol metabolism, may cause injury to the liver,[1,2] as well as to other organs.[3] Numerous in vitro studies have shown that AA can bind covalently to a variety of proteins[4–6] under physiological conditions, resulting in the formation of protein AA-adducts that may interfere with the biological functions of the modified protein.[7–9] A potential role of AA protein adducts in alcohol toxicity has been suggested, because AA also has been shown to bind proteins in vivo.[10–13]

It appears that the epsilon-amino group on lysine and the amino group of N-terminal amino acids are the major sites of AA binding and adduct formation.[4,14,15] The binding of AA to proteins under physiological conditions results in the formation of two types of reaction products that have been classified as unstable and stable adducts. The unstable adducts are mainly Schiff bases, which result from the reaction of a carbonyl carbon with amino groups and which readily dissociate when exposed to dialysis, gel filtration, and treatment with weak acids and bases.[16] However, they can be stabilized by converting them to secondary amines by treatment with the strong reducing agent $NaCNBH_3$. These adducts have been shown to be predominantly ethylated amines and are often referred to as reduced (R) AA adducts.[4,14,16]

Stable adducts, on the other hand, are essentially irreversible products that form in the absence of reducing agents.[16] These adducts are distinctly different than the ethylated amino groups generated by the exposure of Schiff bases to strong reducing agents and are called non-reduced (NR) adducts. It has been suggested that the AA modification of normal proteins by either of these two methods may result in the stimulation of T-cell and antibody responses.[17–21]

Most studies to date have used antibodies with activity to AA adducts that were prepared under conditions favoring the formation of R-AA adducts.[11–13] Additionally, these antibodies are polyclonal and probably recognize more than one AA-protein adduct epitope. The one reported monoclonal antibody to AA-protein adducts is of the IgE isotype,[22] but its utility in immune-based assays may be limited. However, the use of monoclonal antibodies may be useful in detecting the presence or absence of unique AA-protein adducts to determine the role of these adducts in alcohol liver disease. This study reports on a group of monoclonal antibodies that recognize all AA-protein adducts prepared under R conditions. One selected monoclonal antibody is of the IgG2b isotype and can be

From the Alcohol Research Center, Omaha Veterans Administration Medical Center (D.J.T., G.M.T.); and the Departments of Internal Medicine (L.W.K., M.F.S., J.M.D.) and Pathology and Microbiology (T.L.M.), University of Nebraska Medical Center, Omaha, Nebraska.

Received for publication February 1, 1993; accepted June 30, 1993

This study was supported by Grants AA 07818 and AA 04961 from the National Institutes of Alcohol Abuse and Alcoholism and the Department of Veterans Affairs.

Reprint requests: Lynell W. Klassen, M.D., University of Nebraska Medical Center, Department of Internal Medicine, 600 South 42nd Street, Omaha, NE 68198-3332.

Copyright © 1994 by The Research Society on Alcoholism.

PM3006739287

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

easily used to probe for the presence of R AA-protein adducts.

MATERIALS AND METHODS

Antigen Preparation

Bovine brain tubulin was prepared by the method of Shelanski et al.,[23] and rabbit muscle actin was prepared as described by Carsten and Mommaerts.[24] The purity of these proteins was routinely monitored using SDS-PAGE[25] and exceeded 98%. Bovine serum albumin (BSA) (crystallized, lyophilized, and fatty acid free), and keyhole limpet hemocyanin (KLH) were purchased from Calbiochem (San Diego, CA). Mouse plasma proteins, rat plasma proteins, and rabbit plasma proteins were prepared by ammonium sulfate precipitation (70% final) for 4 hr at 4°C and pelleting at 10,000 × *g* in a Beckman JA-21 rotor for 20 min. The supernatants were removed, the pellet resolubilized in double-distilled deionized water, and dialyzed against three changes of phosphate-buffered saline (PBS). All protein concentrations were determined by the method of Lowry et al.[26]

Proteins were labeled with AA using methods previously described.[27] For the preparation of AA-modified proteins under R conditions, 120 μl of 300 mM $NaCNBH_3$ (30 mM final), 60 μl of 100 mM AA (5 mM final), and 20 μl of PBS were added sequentially to 1 ml of protein (1.2 mg/ml in PBS). Both the AA and $NaCNBH_3$ were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used fresh. Proteins were modified under NR conditions by the addition of 120 μl of 50 mM AA (5 mM final) and 80 μl of PBS to 1 ml of protein (1.2 mg/ml). The R protein solution was incubated at 37°C overnight, dialyzed against three changes of PBS for 8 hr, and used for coating ELISA plates or in immunizations. The NR protein solution was incubated at 37°C for 5 days, dialyzed against three changes of PBS for 8 hr, and used as previously described. All antigens were prepared fresh, because storage at 4°C can result in loss of antigenicity (data not shown), perhaps due to cross-linking and precipitation of AA-protein adducts with storage.[28–30] In some assays, crotonaldehyde, butyraldehyde, or propioaldehyde (Aldrich Chemical, Co., Milwaukee, WI) modified proteins were used that were prepared by the same methods as those performed for AA and at the same concentrations.

Monoclonal Antibody Production

Immunizations. Balb/c mice were obtained from the National Cancer Institute via an interagency agreement with the Veteran's Administration and maintained on water and laboratory chow ad libitum. The mice were quarantined immediately on arrival and monitored on a monthly basis to ensure specific pathogen-free status. Five mice were injected intraperitoneally over a period of 6 weeks with 25 μg of tubulin modified with AA under NR conditions in Freund's complete (week 1) or Freund's incomplete (weeks 3 and 5) adjuvant. At weeks 4 and 6, the animals were bled by retro-orbital sinus puncture, serum was obtained and tested against unmodified, NR- and R-AA–modified protein adducts of tubulin, KLH, BSA, and actin in an ELISA.

Fusions. Spleens from animals whose serum showed antibody to NR- and R-AA modified proteins were removed and homogenized in a Stomacher Lab Blender 80 (Tekmar Company, Cincinnati, OH). A single cell suspension was prepared and mixed with an equal concentration of the mouse myeloma P3.NS-1-AG4-1 (ATCC, Bethesda, MD). Using the method of Kohler and Milstein,[31] these cells were fused and put in 96-well plates at 1×10^5 cells/well. Selection was performed using hypoxanthine-aminopterine-thymidine (HAT) followed by hypoxanthine-thymidine (HT) in the media. Wells were monitored for growth and tested for positivity to native, NR, and R proteins using an ELISA system. Positive wells were subcloned twice, using limiting dilution, to ensure monoclonality. Additionally, three separate fusions with over 1500 wells for each fusion were performed to obtain the hybridomas utilized in this study.

Antibody Isotyping

An antigen capture technique was utilized to determine the class and subclass of immunoglobulin of the monoclonal antibodies produced. Briefly, rabbit antimouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (affinity-purified; 1 mg/ml; Zymed Laboratories, Inc., South San Francisco, CA) were diluted 1:500 in bicarbonate buffer (coating buffer), 100 μl added to the appropriate wells of an ELISA plate (Immulon II, Nunc, Fisher Scientific), and allowed to set overnight at 37°C. The plates were then blocked with 200 μl of 1% normal goat serum in PBS-Tween 20 for 2 hr, washed three times with PBS-Tween 20 and monoclonal supernatant (100 μl), or 100 μl of appropriately diluted myeloma proteins (Sigma Chemical Co., St. Louis, MO) was added to the differently coated wells. The plates were incubated at 37°C for 45 min; washed three times with PBS-Tween 20; and 100 μl of alkaline-phosphatase–labeled rabbit anti-mouse IgG, IgM, and IgA (H&L) (Zymed Laboratories) diluted 1:1000 in PBS-Tween 20 was added. Following incubation at 37°C for 45 min, the plates were washed three times with PBS-Tween 20 and 100 μl of substrate was added. The color change was monitored by a Dynatech MicroELISA Reader MR600 (Dynatech, Chantilly, VA) at 405 nm. Wells were considered positive for antibody when the optical density (OD) of test sample was 0.1 OD units greater than when tested on a second antibody control well. This procedure was also utilized to quantitate the concentration of the antibodies in solution by plotting known concentrations of the myeloma proteins against their corresponding OD. Unknown sample concentrations were then extrapolated from their observed OD.

Antibody Activity to Modified Proteins

Unmodified and modified proteins to be tested were diluted to 20 μg/ml in bicarbonate coating buffer (pH 9.6) and the ELISA plates coated overnight at 37°C with 100 μl of the test protein. To the plates, 100 μl of either serum or monoclonal supernatant was added and the plate(s) incubated at 37°C for 45 min. The plates were washed three times with PBS-Tween 20 and 100 μl of alkaline phosphatase-labeled rabbit anti-mouse IgG, IgM, and IgA (H&L) was added. Following a 45-minute incubation at 37°C, the plates were washed three times with PBS-Tween 20 and 100 μl of substrate added. The plates were monitored as described, and wells were considered positive when the difference between the OD on the modified protein and the corresponding native protein was >0.1. Equal binding of unmodified and modified proteins to ELISA plates was confirmed by use of polyclonal antibodies to the unmodified protein. All plates were coated with the same amount of protein.

Preparation of Rabbit Polyclonal Antibody to AA-Protein Adducts

A rabbit polyclonal antibody was made to AA-protein adducts by the method of Worrall et al.[12] Briefly, 8 mg/ml of rabbit plasma proteins was incubated with an equal volume of 480 mM AA for 1 hr at room temperature and $NaCNBH_3$ (100 mM) added for 30 min. The protein was dialyzed overnight, emulsified in Freund's complete adjuvant, and injected intradermally. The animals were boosted twice more over a 4-week period using the same protein adduct prepared previously but in Freund's incomplete adjuvant. The animals were bled and serum tested against our panel of AA-protein adducts, and pair-fed or alcohol-fed rat liver cytosols.

Ethanol-Fed Rat Livers

Male Sprague–Dawley rats (140–150 g) were maintained on a liquid diet containing ethanol as 36% of caloric intake. These rats were pair-fed with rats receiving the liquid control diet, where the ethanol was replaced isocalorically with carbohydrate for up to 8 weeks as previously described.[32] The rats were killed and livers obtained and frozen at −70°C. Cytosol and membrane fractions were prepared by the method of Touster et al.[33] Briefly, liver cytosols were prepared by homogenizing the thawed livers in a Dounce homogenizer with 2–3 ml of PBS. The homogenates

PM3006739288

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

were centrifuged in a Beckman JA-21 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 10,000 × *g* for 20 min to remove membranes. The supernatants were further centrifuged at 100,000 × *g* for 1 hr in a Beckman ultracentrifuge to obtain predominantly cytosolic proteins. Protein content of the supernatants was determined by the method of Lowry et al.[26] and then stored frozen at −70°C until used. One hundred μl of the cytosol proteins at 200 μg/ml was coated onto wells of Immulon II ELISA plates in bicarbonate buffer and used to test antisera or monoclonal antibody reactivities.

Quantification of Aldehyde Adducts on BSA Protein

The number of adducts formed for each aldehyde agent studied was determined by measuring the incorporation of ^3H from $NaCNBH_3$ as previously described.[27] Incubation conditions included 5 mM aldehyde species with 30 mM [^3H]$NaCNBH_3$. The number of adducts formed were relatively expressed as nM ^3H incorporated/mg of protein.

Data Analysis

Data were analyzed using either ABStat (AndersonBell) or SigmaPlot (Jandel Scientific) computer programs. Because relative ODs vary substantially between ELISA assays, the data presented are the average of triplicate wells of a representative experiment. All experiments involving ELISAs have been repeated at least three times with similar results.

RESULTS

Reactivity of Polyclonal Antibody

The antibody titers of mice immunized with NR-modified tubulin to either native, NR, or R tubulin showed no observed differences, because all antibody activity was essentially due to reactivity to native tubulin (Table 1). To avoid the detection of antibody activity specific for the carrier protein (tubulin) and enhance the detection of antibody to the NR and/or R adducts, the antisera were tested using actin, BSA, or KLH as the carrier. As expected, the highest antibody titers were to NR adducts on the protein. However, significant activity to the R adduct (at ~½ the titer to NR) was also observed (Table 1).

Reactivity of Monoclonal Antibody to AA-Modified Proteins

Hybridoma supernatants were screened for activity to native proteins and proteins modified with AA under NR or R conditions. Only activity to the R protein adducts were observed, even though three separate fusions were made and over 4500 potential hybridomas screened. A variety of reaction patterns were observed. Clone 4G12 reacted with only R-tubulin, whereas 1H12 and 4E12 reacted with all proteins prepared under R conditions except tubulin, the carrier protein used for immunization (Table 2). In contrast, the clone 2H10 reacted with only BSA and KLH prepared under R conditions. Thus, there are some clones that appear to show some carrier specificity. However, clone 2D2 was chosen for its broad reactivity to virtually all proteins prepared under R conditions and was subcloned. These subclones of 2D2 were renamed to reflect the antigen detected; RT1.X (*R*educed *T*ubulin 1.X, where X is the number of the subclone).

Table 1. Polyclonal Antibody Response to Proteins Modified with AA Under Reducing and Nonreducing Conditions

	AA-modified proteins		
Antigen	Native	NR	R
Tubulin	>2560	>2560	>2560
Actin	20	1280	320
BSA	10	320	160
KLH	40	640	320

Mice were immunized with NR tubulin. Values represent the reciprocal of the highest dilution that demonstrated a relative OD of >0.100.

Reactivity of Monoclonal Antibodies to Proteins Modified with Other Aldehydes

Four subclones of 2D2 (RT1.1, RT1.2, RT1.3, and RT1.4) were shown to have different activities to tubulin modified with AA when compared to proteins modified with other species (Table 3). All four subclones reacted with AA adducts on tubulin prepared under R conditions with minimal binding to propioaldehyde adducts prepared under these same conditions. None of these subclones reacted to butyraldehyde or crotonaldehyde adducts prepared under R conditions. Similar results were observed when actin, BSA, or KLH were used as carrier proteins. The clone RT1.3 did not react with actin, BSA, or KLH modified with AA under reducing, suggesting this clone was carrier-specific. Similarly, RT1.4 was shown to bind to all proteins modified with AA under reducing conditions, but at a much lesser degree on actin, BSA, and KLH—again suggesting a degree of carrier specificity. RT1.1 and RT1.2 are specific for AA-modified proteins prepared under R conditions and are *not* carrier-dependent (Table 3). RT1.1 was chosen for future studies due to its rapid growth and antibody production rates. Additionally, the reactivity of RT1.1 and RT1.2 to the different aldehydes was dependent on the size of the side chains as evidenced by primary activity on AA minimal activity on propioaldehyde, and no activity to butyraldehyde or crotonaldehyde.

To answer the question of differences in epitope density formed by the various aldehyde species, the amount of ^3H incorporation from $NaCNBH_3$ was determined. As can be seen from Fig. 1, there were no significant differences observed between the number of epitopes formed by each aldehyde. These data demonstrate that the difference in the binding of RT1.1 to various BSA-aldehyde adducts is due to epitope specificity differences and is not simply due to the quantitative changes in adduct number.

Specificity and Sensitivity of RT1.1 to Different Protein Carriers

RT1.1 reacted with a wide variety of proteins modified with AA under R conditions in vitro, further demonstrating that it is not carrier-dependent (Fig. 2). However, it was not possible to assess mouse liver cytosol or plasma, because these preparations had endogenous IgG present

PM3006739289

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

Table 2. Activity of Monoclonal and Rabbit Polyclonal Antibodies to Proteins Prepared Under Reducing and Nonreducing Conditions

MoAb clone no.	Ig isotype	AA modification	Protein assayed: BSA	KLH	Tubulin	Actin
1H12	IgG2b	R	0.120*	0.234	0.000	0.120
		NR	0.004	0.003	0.008	0.010
2D2†	IgG2b	R	0.110	0.134	0.680	0.320
		NR	0.002	0.007	0.006	0.003
2H10	IgG2b	R	0.950	0.100	0.000	0.000
		NR	0.010	0.005	0.008	0.013
4E12	IgG2b	R	0.100	0.140	0.000	0.120
		NR	0.006	0.008	0.010	0.020
4G12	IgG1	R	0.000	0.000	0.150	0.000
		NR	0.000	0.001	0.001	0.003
Rabbit	Polyclonal	R	1.513	1.714	2.000	2.000
		NR	0.936	0.851	1.050	1.236

Monoclonal supernatants from early passage clones were assayed at a 1:4 dilution, the rabbit polyclonal antibody at 1:500. MoAb, monoclonal antibody.
* Relative OD = optical density on the protein-R or protein-NR minus the optical density on the protein-native.
† This clone was chosen and renamed RT1.

Table 3. Activity of Specific RT1 Subclones (2D2) on Aldehyde-Modified Tubulin, Actin, BSA, and KLH Prepared Under Reducing Conditions

Subclone	Isotype	Carrier	Antibody bound to aldehyde-protein adduct: Acetaldehyde (ng)	Propionaldehyde (ng)	Butyraldehyde (ng)	Crotonaldehyde (ng)
RT1.1	IgG2b	Tubulin	1153*	26	<10	<10
		Actin	776	224	<10	<10
		BSA	1295	138	<10	<10
		KLH	1244	380	<10	<10
RT1.2	IgG2b	Tubulin	1026	21	<10	<10
		Actin	1099	273	<10	<10
		BSA	1491	256	<10	<10
		KLH	996	101	<10	<10
RT1.3	IgG2b	Tubulin	1048	<10	<10	<10
		Actin	<10	<10	42	<10
		BSA	<10	<10	<10	<10
		KLH	<10	<10	<10	<10
RT1.4	IgG2b	Tubulin	1031	17	<10	<10
		Actin	212	<10	<10	<10
		BSA	363	<10	<10	<10
		KLH	134	<10	<10	<10

Results are the average of duplicate wells from three different assays.
* 2000 ng of each monoclonal antibody was added to a uniform amount of protein bound to an ELISA plate. Quantification of the amount of antibody bound was determined by extrapolation from a standard curve generated by dilution for the appropriate isotype (mouse IgG2b).

resulting in high backgrounds by the second antibody only. The sensitivity of this assay was determined by coating plates with different concentrations of BSA-R and probing with RT1.1 (Fig. 3). From the standard curve, it was possible to determine that the sensitivity of this assay is as little as 10 ng/ml (8.7 pM of R AA epitope).

The sensitivity of these assays has been increased 2- to 3-fold with a specific second antibody, with no subsequent increase in backgrounds (Fig. 4). This increase in sensitivity will greatly facilitate the detection of R AA adducts on both in vitro and in vivo modified proteins. Additionally, the lack of increases in background greatly enhances the use of this monoclonal antibody. All subsequent studies now utilize this assay system, thereby efficiently detecting RT1.1 in all immune-based assays.

Rabbit Polyclonal Antibody Response to AA-Protein Adducts

The rabbit polyclonal antibody detected both NR and R AA-protein adducts (Table 2). These results would suggest that this polyclonal antibody recognizes several AA-protein adducts and that there are differences between NR and R adducts, because RT1.1 only detects the R adduct, whereas the rabbit polyclonal recognizes both.

Activity of Antibodies to Rat Liver Cytosols

Cytosol preparations of the livers from alcohol-fed rats contained adducts that were detected by a rabbit polyclonal antibody raised against AA-modified proteins (Fig. 5). However, RT1.1 did not detect AA-protein adducts in the cytosols from livers of either alcohol-fed rats or their pair-fed controls. The polyclonal antisera recognizes AA adduct epitopes made under both in vitro R and NR conditions, whereas RT1.1 recognizes an epitope unique to the R AA protein prepared in vitro. This would suggest that at least one AA-protein adduct made under in vitro reducing conditions is not formed in vivo following alcohol ingestion.

To answer the question of whether our assay system was sensitive enough to detect binding of RT1.1 to epi-

PM3006739290

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

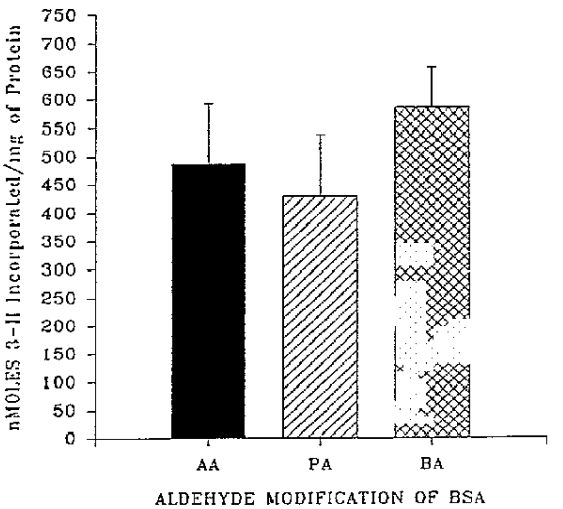

Fig. 1. Relative amount of aldehyde-BSA adducts formed under reducing conditions. The mean ± SE of ^3H incorporation from $NaCNBH_3$ when adducts of BSA were formed with AA, propionaldehyde (PA), or butyraldehyde (BA)/mg under reducing conditions.

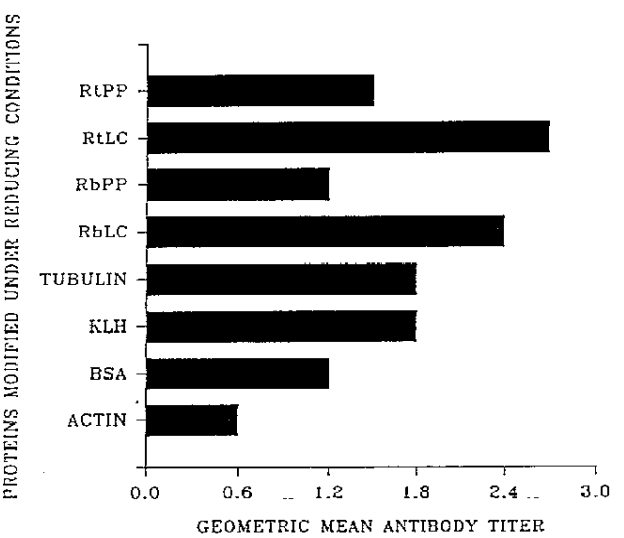

Fig. 2. Reactivity of RT1.1 to different proteins modified in vitro under reducing conditions. The different proteins are plotted against their geometric mean antibody titer from the same lot of purified RT1.1; RtPP, rat plasma protein; RtLC, rat liver cytosol; RbPP, rabbit plasma protein; and RbLC, rabbit liver cytosol.

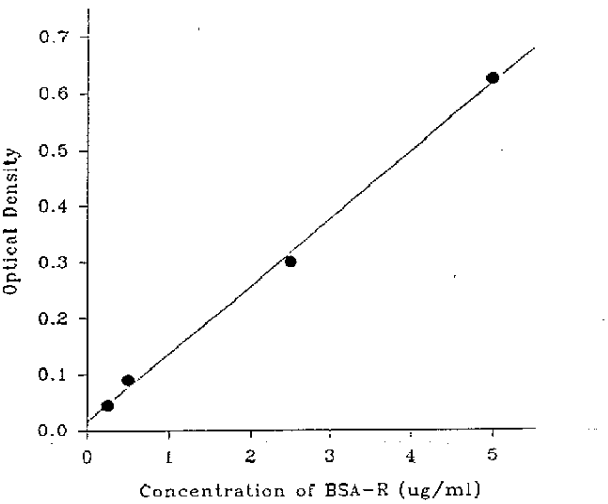

Fig. 3. Reactivity of RT1.1 to ELISA plates coated at different concentrations (μg/ml) with BSA-R. RT1.1 was diluted to 50 mg/ml and the OD recorded against the concentration of BSA-R added to each well. Points represent the average of triplicate wells.

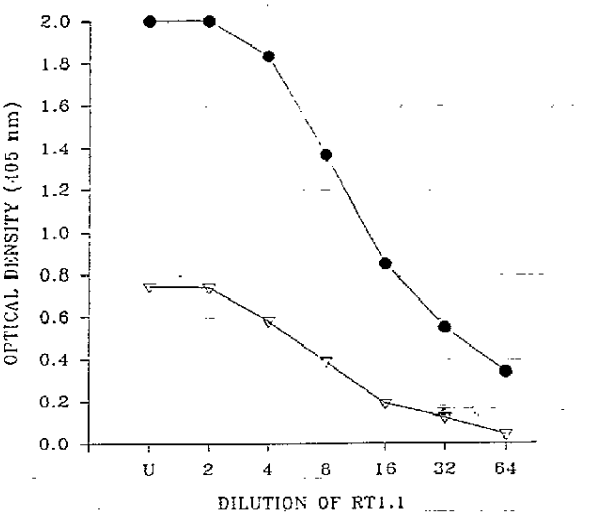

Fig. 4. Activity of purified RT1.1 on BSA-R coated plates (2 μg/well) using alkaline phosphatase-labeled rabbit antimouse IgG, IgM, and IgA (H&L) at 1:1000 dilution (▽) or Rb × MS IgG2b (1:2000) followed by alkaline phosphatase-labeled goat antirabbit IgG (H&L) at 1:1000 dilution (●). The ODs reported are the differences between the activities on BSA-R and BSA native protein. Using an isotype control (IgG2b myeloma), no nonspecific binding was observed.

topes in rat liver cytosols, BSA-R was added at different concentrations prior to coating the plates. By this method it was possible to determine that the minimum detectable level of R-AA–protein adducts in control cytosol was 79 pM of R-AA–epitope. Similarly, 94 pM of R-AA–epitopes was the minimum detectable level when BSA-R was added to alcohol-fed rat liver cytosol. Therefore, these data would suggest that RT1.1 should bind its same epitope if in alcohol-fed rat livers. This is supported by data from alcohol-fed rat liver cytosols spiked with BSA-R that demonstrated the extreme sensitivity of the assay, and the lack of substances that could interfere with RT1.1 binding.

DISCUSSION

These data have shown that it is possible to produce a monoclonal antibody that is specific for proteins modified with AA under the reducing conditions utilized in this study. The RT1.1 clone was chosen because it bound to a broad spectrum of proteins modified with AA under R

PM3006739291

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

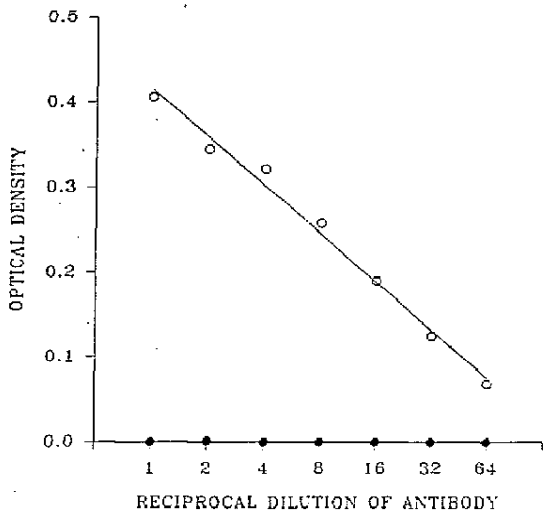

Fig. 5. Reactivity of RT1.1 (●) and a rabbit polyclonal antibody (○) to pair-fed and alcohol-fed rat liver cytosols. The rabbit polyclonal was diluted 1:50 and RT1.1 1:2, and 2-fold dilutions were performed. Differences in the OD reading on alcohol-fed and pair-fed rat livers are plotted against the dilution.

conditions and, thus, is carrier protein-independent. Additionally, in data not shown here, RT1.1 also reacts with proteins modified by methods using higher concentrations of AA and $NaCNBH_3$ utilized by other investigators.[12] Because of its unique specificity, RT1.1 may be utilized to characterize better the types of adducts formed when AA binds to proteins under other conditions of chemical reduction.

Other monoclonal antibody clones were produced that recognized different epitopes associated with R adducts. For example, the clone 4G12 recognized only actin-R adducts and not tubulin-R. Thus, some AA-protein adduct epitopes appear to be carrier-specific, whereas others are not. Another problem evident in the specificity pattern of clone 4G12 is that the antibody does not bind to the immunizing antigen (tubulin adducts). Possible explanations could include masking of the tubulin-AA adduct epitopes during the binding to the ELISA plate, or significant differences in epitope density between actin-AA adduct and the tubulin-AA adduct conjugates. Another explanation is suggested by recent data that show antibodies to the R adducts exist naturally in humans, horses, cows, rats, and mice (unpublished observations). This observation is consistent with earlier studies that demonstrated the presence of low levels of antibodies to proteins modified under R conditions in normal human controls and at higher levels in patients with alcohol liver disease.[34–36] Therefore, it is possible that some of these hybridomas could have been produced by the fusion of a naturally occurring B-cell having specificity to R adducts with the myeloma cell line. Nevertheless, the strength of the RT1.1 monoclonal antibody is its broad reactivity with all proteins modified with AA under at least two different in vitro R conditions.

One of the most surprising findings was that RT1.1 did not recognize the original NR protein adduct the mouse was immunized with. An explanation for the production of an antibody to a R adduct following immunization with NR-tubulin may be that the R-AA–epitope is present on proteins prepared under NR conditions. However, this is probably not the case, because RT1.1 never binds to any protein modified under NR conditions. Furthermore, the sensitivity testing performed in this study suggests that, if the epitope was present, it should have been detected by this antibody. The use of isotype-specific secondary antisera (rabbit anti-IgG2b) has increased the sensitivity of the ELISA 2- to 3-fold without any detectable binding of RT1.1 to NR proteins. However, the rabbit polyclonal antibody we developed detected both NR and R adducts in vivo, demonstrating that the NR- and R-AA adducts are very distinct from one another and that only one adduct species (NR or R) is made under the conditions used herein.

Another possibility is that the NR adduct may be modified in vivo to express epitopes seen on R-AA–protein adducts. Although Tuma et al.[16] have shown that the NR-AA adduct is stable and cannot be readily modified in vitro by the reducing agent $NaCNBH_3$, this does not necessarily mean that modification does not occur by macrophages during antigen processing or presentation. Hepatocytes or Kupffer cells could also modify the NR-AA adduct to look like the R-AA by some undescribed mechanism.

The finding that a monoclonal antibody could not be produced to the NR protein adducts, when a polyclonal antibody could be, is of extreme importance. This occurred despite performing three different fusions (4500 wells). It is possible that there was poor fusion rates or that the clones were unstable. However, from each of these three fusions, large numbers (50–100) of hybridomas producing antibodies that recognized the R AA-protein adduct were generated. It would seem unlikely that only the hybridomas that produced antibodies to the NR-AA–protein adduct were unstable. It is more probable that there is more than one type of AA adduct formed under NR conditions, each with a low epitope density on the modified protein. The polyclonal response would detect all of these adducts giving a positive result by ELISA. Monoclonal antibodies may only bind a small percentage of the total number of epitopes and thereby avoid detection in the in vitro assay system. Further development and characterization of monoclonal and polyclonal antibodies that recognize NR adduct epitopes are currently under investigation.

Primary work by other investigators have utilized rabbit polyclonal antibodies and demonstrated the presence of an antigen in the livers of alcohol-fed rats, but not in pair-fed controls.[12,37,38] Lin et al.[37] have shown the presence of a 37 kDa liver protein adduct that appears to form in vivo during chronic alcohol ingestion when using their anti-

PM3006739292

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

bodies. Other investigators have shown the presence of P-45011E1 adduct.[38] In contrast, Worrall et al.[12] have shown their antibody will bind to a large number of proteins in alcohol-fed rat livers. In these experiments presented herein, a rabbit polyclonal was prepared in a similar method as Worrall et al.[12] This antibody reacted with alcohol-fed rat liver cytosols and not pair-fed controls in an ELISA system. However, RT1.1 did not react in this system and would suggest that the reduced AA-protein adduct it recognizes is not present. This information would be interpreted to mean that the R-AA adduct produced under two different conditions, and capable of being detected on any protein modified in this manner, is not present following chronic alcohol ingestion in rats.

This is not due to a sensitivity problem as studies reported herein suggest the limits of detection by RT1.1 to be as low as 79 pM of R-AA–epitope.

This study reports on a monoclonal antibody to proteins modified with AA under R conditions. Its method of production and the results shown herein suggest that the problem of detecting protein-AA adducts is more complex than originally anticipated. It has become apparent that there is more than one type of AA adduct present in most of the in vitro preparations previously reported in the literature. The use of monoclonal antibodies that recognize unique and specific AA-adduct species will allow new approaches to the study of alcohol-induced tissue damage by documenting which types of adducts are present or absent following modification with AA under different in vitro and in vivo conditions.

ACKNOWLEDGMENTS

The authors would like to thank Susan Lemley-Gillespie and Kirk Wegter for their diligence and expertise in performing the assays utilized in this study. Additionally, the patience and diligence of Shirley Yeich in the preparation of this manuscript was greatly appreciated.

REFERENCES

1. Jennett RB, Tuma DJ, Sorrell MF: Effects of acetaldehyde on hepatic proteins, in Popper H, Schaffner F (eds): Progress in Liver Diseases, vol IX. Philadelphia, Saunders, 1990, pp 325–333

2. Lieber CS: Hepatic, metabolic and toxic effects of ethanol: 1991 update. Alcohol Clin Exp Res 15:573–592, 1991

3. VonWartburg JP: Acute aldehyde syndrome and chronic aldehydism. Mutat Res 186:249–259, 1987

4. Donohue TM, Tuma DJ, Sorrell MF: Acetaldehyde adducts with proteins: Binding of [^{14}C]acetaldehyde to serum albumin. Arch Biochem Biophys 220:239–246, 1983

5. Stevens VJ, Fantl WJ, Newman CB, Sims RV, Cerami A, Peterson CM: Acetaldehyde adducts with hemoglobin. J Clin Invest 67:361–369, 1981

6. Mauch TJ, Donohue TM, Zetterman RK, Sorrell MF, Tuma DJ: Covalent binding of acetaldehyde selectively inhibits the catalytic activity of lysine-dependent enzymes. Hepatology 6:263–269, 1986

7. Smith SL, Jennett RB, Sorrell MF, Tuma DJ: Acetaldehyde substoichiometrically inhibits bovine neurotubulin polymerization. J Clin Invest 84:337–341, 1989

8. Tuma DJ, Smith SL, Sorrell MF: Acetaldehyde and microtubules. Ann NY Acad Sci 625:786–792, 1991

9. Jennett RB, Saffari-Fard A, Sorrell MF, Smith SL, Tuma DJ: Increased covalent binding of acetaldehyde to calmodulin in the presence of calcium. Life Sci 45:1461–1466, 1989

10. Medina VA, Donohue TM, Sorrell MF, Tuma DJ: Covalent binding of acetaldehyde to hepatic proteins during ethanol oxidation. J Lab Clin Med 105:5–10, 1985

11. Lin RC, Smith RS, Lumeng L: Detection of a protein-acetaldehyde adduct in the liver of rats fed alcohol chronically. J Clin Invest 81:615–619, 1988

12. Worrall S, DeJersey J, Shanley BC, Wilce PA: Detection of stable acetaldehyde-modified proteins in the livers of ethanol-fed rats. Alcohol 26:437–444, 1991

13. Niemela O, Juvonen T, Parkkila S: Immunohistochemical demonstration of acetaldehyde-modified epitopes in human liver after alcohol consumption. J Clin Invest 87:1367–1374, 1991

14. Tuma DJ, Newman MR, Donohue TM, Sorrell MF: Covalent binding of acetaldehyde to proteins: Participation of lysine residues. Alcohol Clin Exp Res 11:579–584, 1987

15. San George RC, Hoberman HD: Reaction of acetaldehyde with hemoglobin. J Biol Chem 261:6811–6821, 1986

16. Tuma DJ, Hoffman T, Sorrell MF: The chemistry of acetaldehyde-protein adducts. Alcohol Alcohol Suppl 1:271–276, 1991

17. Johnson RD, Williams R: Immune responses in alcoholic liver disease. Alcoholism 10:471–486, 1986

18. MacSween RNM: Alcohol and liver injury: Genetic and immunologic factors. Acta Med Scand (Suppl.) 703:57–67, 1986

19. Zetterman RK, Sorrell MF: Immunologic aspects of alcoholic liver disease. Gastroenterology 81:616–624, 1981

20. Orrego H, Israel Y, Blendis IM: Alcoholic liver disease: Information in search of knowledge. Hepatology 1:267–283, 1981

21. Tuma DJ, Klassen LW: Immune responses to acetaldehyde-protein adducts: Role in alcoholic liver disease. Gastroenterology 103:1969–1973, 1992

22. Israel Y, Hurwitz F, Niemela O, Arnon R: Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. Proc Natl Acad Sci USA 83:7923–7927, 1986

23. Shelanski ML, Gaskin F, Cantor CR: Microtubule assembly in the absence of added nucleotides. Proc Natl Acad Sci USA 70:765–768, 1973

24. Carsten ME, Mommaerts WFHM: A study of actin by means of starch gel electrophoresis. Biochemistry 2:28–32, 1963

25. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970

26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951

27. Klassen LW, Xu DS, Tuma DJ: Immune response to acetaldehyde adducts, in Seminare D, Watson RR, Pawloski A (eds): Alcohol, Immunosuppression and AIDS. New York, Alan R. Liss, Inc., 1990, pp 333–340

28. Gaines KC, Salhany JM, Tuma DJ, Sorrell MF: Reactions of acetaldehyde with human erythrocyte membrane proteins. FEBS Lett 75:115–119, 1977

29. Kikugawa K, Takayanagi K, Wantanabe S: Polylysines modified with malonaldehyde, hydroperoxylinoleic acid and monofunctional aldehydes. Chem Pharm Bull 33:5437–5444, 1985

30. Kikugawa K, Iwata A, Beppu M: Formation of cross-links and fluorescence in polylysine, soluble proteins and membrane proteins by reaction with 1-butanal. Chem Pharm Bull 36:685–692, 1988

31. Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–500, 1975

32. Casey CA, Kragskow SL, Sorrell MF, Tuma DJ: Chronic ethanol administration impairs the binding and endocytosis of asialo-orosomucoid in isolated hepatocytes. J Biol Chem 262:2704–2710, 1987

33. Touster O, Arensen NN Jr, Dulaney JT, Hendrickson H: Isolation of rat liver plasma membranes: Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. J Cell Biol 47:604–618, 1985

PM3006739293

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001

34. Niemela O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y: Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology 7:1210–1214, 1987

35. Hoerner M, Behrens UJ, Worner TM, Blacksbert I, Braly LF, Schaffner F, Lieber CS: The role of alcoholism and liver disease in the appearance of serum antibodies against acetaldehyde adducts. Hepatology 8:569–574, 1988

36. Worrall S, DeJersey J, Shanley BC, Wilce PA: Antibodies against acetaldehyde-modified epitopes: Presence in alcoholic, non-alcoholic liver disease and control subjects. Alcohol Alcoholism 25:509–517, 1990

37. Lin RC, Smith RS, Lumeng L: Detection of a protein-acetaldehyde adduct in the liver of rats fed alcohol chronically. J Clin Invest 81:615–619, 1988

38. Behrens UJ, Hoerner M, Lasker JM, Lieber CS: Formation of acetaldehyde adducts with ethanol-inducible P45011E1 in vivo. Biochem Biophys Res Commun 154:584–590, 1988

PM3006739294

Source: https://www.industrydocuments.ucsf.edu/docs/mtnx0001